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# Transcription of human 7S K DNA *in vitro* and *in vivo* is exclusively controlled by an upstream promoter

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ABSTRACT

We have analyzed the transcription of a recently isolated human 7S K RNA gene in vitro and in vivo. In contrast to hitherto characterized class III gens (genes transcribed by RNA polymerase III), the coding sequence of this gene is not required for faithful and efficient transcription by RNA polymerase III. In fact, a procaryotic vector DNA sequence was efficiently transcribed by RNA polymerase III under the control of the 7S K RNA gene upstream sequence in vitro and in vivo. $S_1$-nuclease protection analyses confirmed that the 7S K 5'flanking sequence was sufficient for accurate transcription initiation. These data demonstrate that 7S K DNA represents a novel class III gene, the promoter elements of which are located outside the coding sequence.

## INTRODUCTION

Several low molecular weight RNA species such as 5S and tRNA (1), small viral RNAs (2,3) as well as the small cytoplasmic RNA (scRNA) species 7S K and L (4,5) have been designated as class III genes, since they are transcribed by RNA polymerase III. In addition, in contrast to other small nuclear RNA (snRNA) species (6,7), snRNA $U_6$ also is transcribed by RNA polymerase III (8).

The RNA polymerase III transcription system is characterized by gene internal promoter elements. 5S RNA genes are regulated by a continuous internal control region (icr;9,10,11), whereas tRNA and VA RNA genes are controlled by an internal split promoter (12,13,14,15,16). The A-box and B-box elements of such split promoters have been characterized by consensus sequences (17) and an A-box like sequence element has also been identified within the icr of the 5S RNA gene (18,19). In addition to the

internal promoter elements, 5'flanking sequences have been found to modulate the activity of several tRNA genes (20,21). This holds true also for the two human 7S RNA genes which have been isolated recently (22,23,24). Accurate and efficient transcription of human 7S L RNA depends on a sequence element located around position -20 to -40 (22) whereas comparable control regions have been identified within human 7S K DNA between position -60 and the transcription start site (24,25).

In this study we analyzed the gene internal promoter elements of human 7S K DNA. By transcription experiments performed with a series of internal deletion mutants and with fusion genes between the 5'upstream region of human 7S K DNA and a procaryotic vector sequence as coding part, we demonstrate that the 5'flanking part of the human 7S K DNA alone is suffcient for efficient transcription initiation in vitro and in vivo.

## MATERIALS AND METHODS

Reagents and Enzymes: Restriction enzymes and mung bean nuclease were obtained by Pharmacia; Klenow Fragment of DNA polymerase I, T4 DNA ligase and α-amanitin by Boehringer Mannheim; dNTPs and rNTPs by Sigma; $\alpha$-$^{32}$P UTP and $\gamma$-$^{32}$P ATP by New England Nuclear. Electrophoresis reagents were obtained by Serva (Heidelberg), all other chemicals were from Merck.

Deletion mutants and fusion genes: Internal deletion mutants were obtained after restriction and religation of the sites indicated within the wild type gene. Ligation of noncompatible ends was achieved after filling with the Klenow enzyme or removing protruding ends with mung bean nuclease. Construct I (Fig.3) was obtained by insertion of the blunt ended Eco RI - Eco RV pAT 153 fragment into the filled Acc I site of the 7S K wild type gene. Fusion gene II was constructed by first cloning the Pst I - Kpn I fragment of the wild type gene into the pUC 18 vector. Subsequently the fragment was recovered by restriction with the Pst I and Eco RI enzymes and inserted into the respective sites of the pAT 153 vector. From this plasmid a Pst I - Eco RV (blunt) fragment was recovered and cloned together with the Hinf I (filled)-Eco RI fragment (3'end) of the 7S K RNA gene into the pUC8 vector restricted with Eco RI and Pst I.

In vitro transcription and analysis of RNA: Cytoplasmic S100 extracts were prepared as described by Weil et al.(26). In vitro transcription reactions were performed as described in detail previously (24) with 1.5µg of plasmid DNA as template in a total volume of 50 µl. All reactions contained 1 µg of α-amanitin. Phenol extracted RNA was analyzed by electrophoresis in 6% polyacrylamide gels in the presence of 8 M urea.Gels were exposed to Fuji X-ray films for 16 hours using Cronex intensifier screens.

$S_1$-nuclease protection analysis: $S_1$-nuclease protection experiments were performed with unlabeled RNA hybridized to labeled DNA fragments. In vitro transcription was terminated by addition of RNase-free DNAse I ((0.4 mg/ml) and incubation for 30 min. at 30°C. After phenol extraction of the RNA, hybridization was in 80% formamide; 40 mM Pipes, pH 6.4; 0.4 M NaCl and 1 mM EDTA in a total volume of 20 µl for 15 hours at 52°C. $S_1$-nuclease digestion was after addition of 300 µl $S_1$-buffer (0.28 M NaCl; 30 mM Sodium acetate, pH 4.5; 4.5 mM zinc acetate; 30 µg/ml herring sperm DNA and 300 u/ml $S_1$-nuclease) for 15 min. at 37°C. The reaction was terminated by addition of 0.37 M ammonium acetate - 7.5 mM EDTA and the phenol extracted RNA analyzed as above. Protected fragments obtained with in vitro synthesized RNA were analyzed in comparison to the sequencing reactions obtained with the labeled DNA by the method of Maxam and Gilbert (27).

Transformation of HeLa cells: $1.2 \times 10^6$ HeLa cells were transfected with 20 µg of plasmid DNA by the calcium phosphate DNA coprecipitation method (28) as modified by Gorman et al. (29). After 42 hours, nuclear and cytoplasmic RNA was isolated as described previously (30) and hybridized to the labeled DNA fragments indicated in Fig. 5. $S_1$-nuclease digestion and analysis of protected fragments was as above.

## RESULTS

In vitro transcription of 7S K RNA deletion mutants: To determine which sequences inside the human 7S K RNA gene are essential for expression, we introduced deletions into the coding sequence at the restriction sites indicated in Fig 1.

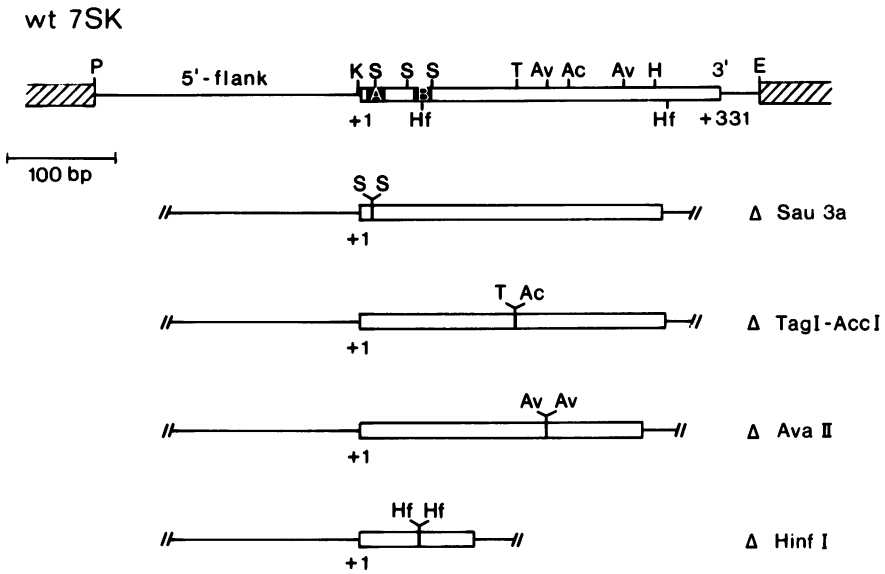


Figure 1. Restriction maps of 7S K DNA clones used for in vitro transcription: Deletion mutants were obtained from the 7S K RNA wild type gene (wt 7SK) after restriction and religation of the sites indicated. Abbreviations for restriction enzymes are: Pst I(P);Kpn I(K);Sau 3A(S);Hinf I(Hf);Taq I(T);Ava II(Av);Acc I(Ac);Hind III(H);Eco RI (E). The A-box and B-box equivalents are indicated by filled boxes within the coding sequence (open segment). Position +1 marks the transcription start site and +331 the 3'end of the gene.

These internal deletions consisted in removal of the coding sequence between the two restriction sites indicated for each mutant. The deletion mutants were analyzed for template activity by in vitro transcription experiments with cytoplasmic S-100 extract. The patterns of in vitro RNA synthesis obtained with supercoiled plasmid DNA of these mutants in comparison to the wild type gene are shown in Figure 2.

Deletion of the sequence between the first (position +12) and the third (+63) Sau 3A restriction site of the coding sequence eliminated the B-box equivalent of the gene. However, since religation of these two Sau 3A sites reconstituted a fairly good A-box homology (compare the sequence in Fig.3 of ref.24), a second kind of Sau 3A deletion mutant was obtained by removing the protruding ends of both Sau 3A sites with mung bean nuclease prior to religation. Thus, this construct did not contain an A-

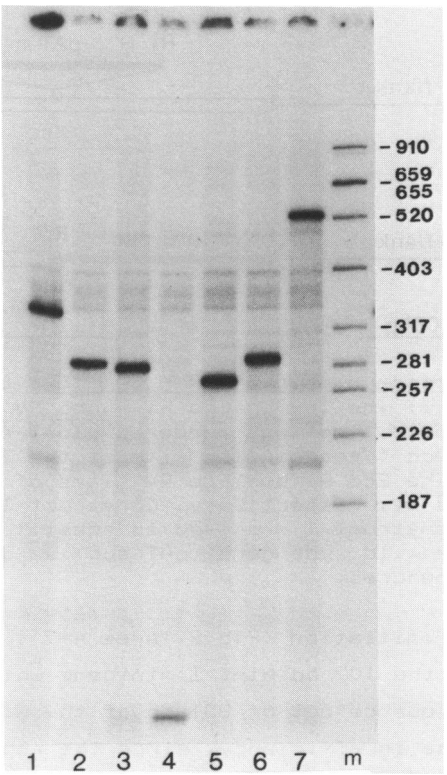


Figure 2. In vitro transcription patterns obtained with wild type or mutant DNA of the 7S K RNA gene: In vitro transcription, purification and analysis of RNA was as described in Experimental Procedures. Templates used were supercoiled 7S K wild type DNA (lane 1) or deletion mutants: lane 2=Sau 3A (+12 to +63); lane 3=same as 2 but protruding ends were removed prior to religation to prevent reconstitution of an A-box; lane 4=Hinf I (+55 to +279); lane 5= Ava II (+169 to +239); lane 6=Taq I-Acc I (+139 to + 190); lane 7=insertion construct I of Fig.3. M=labeled DNA fragments as relative molecular mass markers.

box equivalent anymore. As is seen from lane 2 and 3 of Figure 2, in vitro transcription of these mutants produced the expected 280 nt long transcripts with a comparable efficiency to that of the wild type gene (lane 1). The three other deletions were obtained by removing the sequences between the Taq I (+139) and the Acc I (+190) sites, between the two Ava II (+169 and +239) or the two Hinf I (+55 and +279) restriction sites, respectively. All these deletions did not affect the efficiency

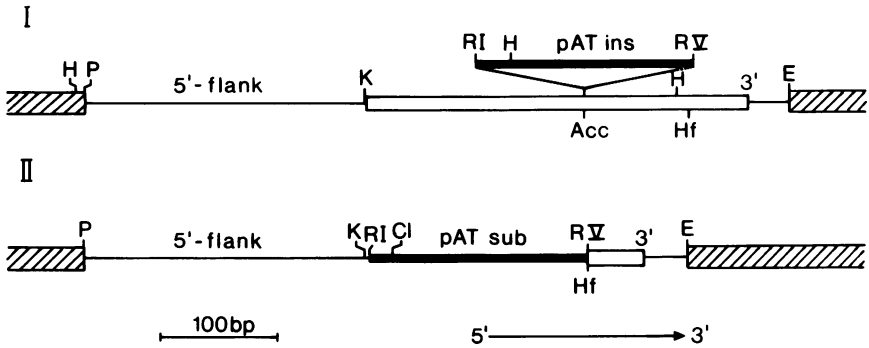


Figure 3. Restriction maps of 7S K - pAT 153 vector DNA fusion genes: Construct I: The 189 bp Eco RI - Eco RV fragment of pAT 153 vector DNA (bold line) was rendered blunt-ended and inserted into the filled Acc I restriction site of the 7S K RNA wild type gene (pAT ins). The Eco RI and Eco RV sites of the vector DNA are marked RI and RV, respectively. Construct II: The same fragment as in construct I was used to substitute the first 279 base pairs of the wild type gene (pAT sub) as described in Experimental Procedures.

of transcription initiation (Fig.2,lanes 4-7). Even transcription of the 107 bp Hinf I minigene which lacks the central 224 nt sequence out of 331 bp of the wild type gene showed no decrease in efficiency, since the reduced intensity of the transcript observed in lane 4 corresponded to the reduced transcript size. Likewise, a maxigene obtained by insertion of 189 base pairs of procaryotic vector sequence of pAT 153 into the Acc I site (see construct I of Fig.3) was as active as the wild type gene itself (Fig.2,lane 7). Together, these results demonstrated that no part of the coding sequence was essential for transcription of the human 7S K RNA gene.

Analysis of 7S K - pAT 153 fusion genes: Consequently, we analyzed whether the upstream sequence of the human 7S K RNA gene directs by itself RNA polymerase III transcription, even of a completely unrelated DNA sequence. For these experiments, the first 279 base pairs of the 7S K coding sequence were replaced by the 189 bp Eco RI - Eco RV fragment of the pAT 153 vector (see construct II of Fig.3). A second construct contained a duplicated plasmid sequence between the 5'flank and the 3'end of 7S K DNA. This construct was obtained by inserting a second Eco RI - Eco RV fragment into the Cla I site of construct II. Thus,

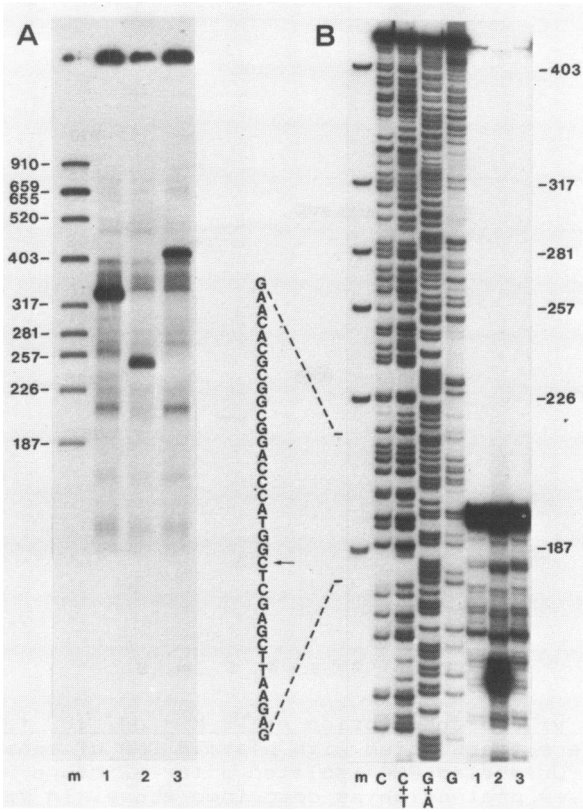


Figure 4. In vitro transcription of 7S K - pAT 153 fusion genes and $S_1$-nuclease protection analysis of the RNA: A: Patterns of in vitro RNA synthesis obtained with different templates. Lane 1=7S K RNA wild type gene; lane 2=construct I of Fig.3 as template; lane 3=maxigene of construct I as template. This plasmid was obtained by insertion of a second pAT 153 Eco RI - Eco RV fragment into the filled Cla I (Cl, +24 of pAT 153) restriction site of the first pAT 153 fragment of construct I. B: $S_1$-nuclease protection analysis of the transcripts of lane 2 of part A (lane 1) or lane 3 of part A (lane 2). The second protected fragment in lane 2 was due to the duplicated vector sequence. Lane 3 shows the protection pattern of transcripts obtained with an intermediate fusion of construct I. This plasmid contained the 5'flanking sequence of 7S K DNA cloned into the Pst I-Eco RI sites of pAT 153 vector.Thus, this plasmid did not contain the 3'end of the 7S K gene and therefore lacks a termination signal.This plasmid containing the integer Eco RV site of the pAT 153 vector sequence was 5'end-labeled at the Eco RV site with $\gamma$-$^{32}$P-ATP using poly-nucleotide kinase. The sequence of the coding strand of this fragment is presented on the left with the arrow indicating the transcription start site.

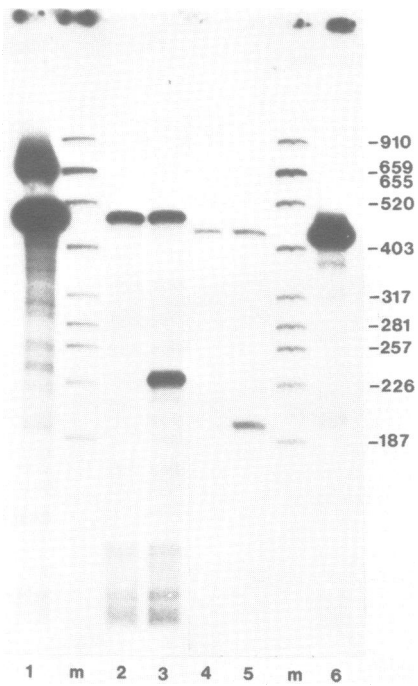


Figure 5. In vivo transcription of 7S K - pAT 153 fusion genes: HeLa cells were transfected with plasmid DNA of construct I and II of Fig.3. Unlabeled RNA isolated after 42 hours was analyzed by $S_1$-nuclease protection as described above. In case of construct I, a 460 bp Hind III fragment (lane 1) was hybridized to RNA from control cells (lane 2) or transfected cells (lane 3). Transcripts originating from transfection with construct II were analyzed with the 430 base pairs Pst I - Eco RV fragment (lane 6) already used in Fig.4. RNA was from control cells (lane 4) or transfected cells (lane 5).

both constructs represented a procaryotic DNA sequence fused to the 7S K upstream promoter element and supplemented with the 7S K transcription termination signal (31). Transcription of these plasmids showed that both constructs were efficiently transcribed *in vitro* (Fig.4A, lanes 2 and 3), though sligthly reduced if compared to the 7S K RNA wild type gene (Fig.4A,lane 1).

Determination of transcription start sites: To further characterize these transcripts, $S_1$-nuclease protection experiments were performed with RNA obtained with both

constructs as template. The comparison of the protected bands (Fig.4B,lanes 1 and 2) with the Maxam-Gilbert sequencing reactions of the coding strand of the labeled fragment (left side of Fig.4B) identified the start nucleotide of both *in vitro* synthesized RNAs. In consideration of the 1.5 nt deviation observed between chemically sequenced and nuclease digested DNA (32), this transcription start site is at the first G of vector DNA (arrow). Compared to the authentic gene, this corresponds to position -1. A modified construct II lacking the 7S K termination sequence (Eco RV-Eco RI fragment of construct II) did not show an alteration of accuracy and efficiency of transcription (Fig 4B,lane 3). In summary, the 5'flanking sequence of the human 7S K RNA gene alone is sufficient to direct accurate and efficient transcription by RNA polymerase III *in vitro*.

*Expression of 7S K - pAT 153 fusion genes in vivo:* One might argue that these *in vitro* transcription results do not necessarily reflect functionality *in vivo*. Such a demonstration, however, is particularly important in case of the RNA polymerase III transcription system since this enzyme reveals a strong tendency for arteficial transcription of a variety of procaryotic vector sequences or mammalian Alu-sequences. In contrast, such sequences are not independently transcribed by RNA polymerase III in vivo. Therefore, HeLa cells were transfected with the plasmid constructs I and II shown in Figure 3. RNA was isolated from transfected cells after 42 hours. It was analyzed for specific 7S K fusion gene transcripts. In both cases, the isolated RNA protected labeled DNA fragments of the expected size as seen in Figure 5, lane 3 (construct I) and lane 5 (construct II). In contrast, no such protected fragments were observed with RNA from control cells (Fig. 5, lanes 2 and 4),respectively. It should be noted that both analyses were performed with DNA labeled within the procaryotic part of the sequence. These results demonstrated that also in intact HeLa cells, the upstream promoter alone of the 7S K RNA gene directed transcription of a procaryotic sequence. No difference in fusion gene activity was observed irrespective whether the procaryotic sequence was an additional part of the human 7S K RNA gene or

replaced the first 83% of the wild type coding region including the authentic transcription start site.

## DISCUSSION

In recent years, the mechanism of transcription of low molecular weight RNA species by RNA polymerase III has been studied extensively. With the availability of a variety of different class III genes, some general characteristics of the RNA polymerase III transcription system have evolved. First, regulation of all class III genes studied so far depends on the activity of gene internal promoters. These internal promoters may (tRNA and 7S L RNA genes; 21,20,22) or may not (5S RNA genes) be modulated by external regulatory elements. Furthermore, the A-box homology has been identified as a common sequence shared by most of these class III promoters (18). In contrast, other conserved blocks such as the B-box and the C-box seem to represent gene specific regulatory elements (12,13,10). A similar picture has emerged by studying RNA polymerase III transcription factors. In addition to the gene specific transcription factor TFIIIA of the 5S RNA gene (11), two common protein factors (TFIIIB and TFIIIC) have been identified for transcription of class III genes (33). Both, the A-box (34) and the B-box (35) elements were found to influence the binding of TFIIIC to class III genes, which in turn is required for binding of TFIIIB. Stable association with TFIIIB has been identified as the rate-limiting step in transcription initiation of 5S RNA genes (36,37).

Thus, it appears that common and gene specific sequence elements in conjunction with common and gene specific transcription factors are involved in regulating transcription initiation of class III genes.

When initially analyzed, the human 7S K RNA gene appeared to be in perfect agreement with these characteristics of the RNA polymerase III transcription system (23,24). The A-box (+9 to +18) and the B-box (+49 to +58) were found properly positioned within the coding sequence, suggesting a tRNA type split promoter. In addition, transcription of the gene was strictly dependent on the presence of the upstream region with an

essential sequence element located between position -60 and -25 (24,25).

During our attempts to determine the gene internal promoter elements it became evident, however, that human 7S K DNA represents a different type of class III gene. Any minigene or maxigene construct was acceptable for efficient initiation of transcription. Internal deletions removing the otherwise important A and B-box like sequences did not show any decrease in transcription initiation efficiency of these mutant genes. Furthermore, full replacement of the coding sequence by a procaryotic vector DNA did not result in a loss of gene activity. These functional analyses revealed that the upstream sequence of human 7S K DNA is exclusively required for accurate and efficient transcription initiation both *in vitro* and *in vivo*.

We conclude that among the eucaryotic genes transcribed by RNA polymerase III, the human 7S K RNA gene is a novel type in terms of the absence of any gene internal promoter function including the A and B-boxes. Comparable conclusions were reached in a recent publication by Murphy et al.(25), though their data were obtained by *in vitro* transcription experiments only.

Recent results concerning the regulatory elements of a $U_6$ RNA gene (38,39) established an essential function for the RNA polymerase II-enhancer like octamer motif ATTTGCAT, found upstream of this $U_6$ RNA gene. Two related octamer motifs are found upstream of the 7S K RNA coding sequence at positions -230 and -130. It is questionable, however, whether these elements are also important for 7S K transcription, as concluded by Murphy et al.(25) from the transcription efficiency of their -59 deletion mutant. Our previous results (24) had shown that two deletion mutants (-111 and -67), although lacking these octamer motifs, both show essentially wild type gene activity when analyzed by *in vitro* transcription.

In view of these findings, the question arises whether the general class III gene transcription factors TFIIIB and TFIIIC also play a role in transcription initiation of these human genes transcribed by RNA polymerase III. Further studies will have to analyze the number and identity of protein factors

required in addition to RNA polymerase III for faithful transcription of these genes. It is quite conceivable that aside from the RNA polymerase itself, none of the general class III gene sequence elements and transcription factors identified so far is involved in synthesis of 7S K and $U_6$ RNA.
Thus in general, class III genes are not necessarily characterized by gene internal promoters. It rather appears that eucaryotic genes transcribed by RNA polymerase III represent a spectrum of at least three different promoter types: i. the 5S RNA gene is exclusively regulated by the internal control region; ii, tRNA, VA RNA and 7S L RNA genes are controlled by a cooperation between external and gene internal regulatory elements; iii. finally, transcription of 7S K RNA solely is controlled via an upstream promoter.

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